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Tissue-Fractionation Studies

14. THE ACTIVATION OF LATENT DEHYDROGENASES IN MITOCHONDRIA FROM RAT LIVER*

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(*Received* 5 *August* 1959)

Several mitochondrial enzymes are known to occur in a latent form, for example adenosine triphosphatase (Kielley & Kielley, 1951), glutamic dehydrogenase (Hogeboom & Schneider, 1953), aconitase (Dickman & Speyer, 1954), cytochrome *c* reductases (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955; Borgström, Sudduth & Lehninger, 1955), cytochrome oxidase (Simon, 1958), rhodanese (Greville & Chappell, 1959) and alkaline deoxyribonuclease (Baudhuin, 1959). The activities of the enzymes in intact mitochondria towards added substrates are relatively low and can be increased, sometimes tenfold or more, by damage to the particles. The acid hydrolases which are localized in the lysosomes of rat liver behave in a similar manner (de Duve *et al.* 1955). These enzymes can be liberated into solution and their activities revealed by various procedures such as incubation at 37°, freezing and thawing, treatment in a Waring Blendor, osmotic shock and treatment with detergents and certain enzymes (Berthet & de Duve, 1951; Berthet, Berthet, Appelmans & de Duve, 1951; Appelmans & de Duve, 1955; Gianetto & de Duve, 1955; Wattiaux & de Duve, 1956; Beaufay & de Duve, 1959). In graded activation experiments the activities of the various hydrolases appear concurrently. There is good evidence that the latency of lysosomal enzymes is due to the inability of the substrates to penetrate the membrane of the particle, and that the apparent activation is due to rupture of the membrane and release of the enzymes into solution.

This paper describes the results of a parallel study of the release of enzymes associated with the two types of particle. Acid phosphatase was taken as being a typical lysosomal enzyme and glutamic dehydrogenase was chosen to represent mitochondria. Some experiments were also done with malic and D-β-hydroxybutyric dehydrogenases. A brief account of the results has already been given (Bendall, 1958), and evidence that these dehydrogenases belong to the true mitochondria has been described (Beaufay, Bendall, Baudhuin & de Duve, 1959; Beaufay, Bendall, Baudhuin, Wattiaux & de Duve, 1959).

EXPERIMENTAL

Methods

All experiments were performed on a combined heavy and light mitochondrial fraction (which contains most of the lysosomes as well as the mitochondria) isolated in 0·25 M-sucrose from rat liver by the method described by Appelmans, Wattiaux & de Duve (1955). The particles were washed once, resuspended in 0·25 M-sucrose (at a concentration corresponding to 0·5–1·0 g. of original liver/ml.) and stored at 0° before use. In a few experiments the preparation and suspension medium contained mM-ethylenediaminetetra-acetic acid (EDTA), pH 7·7.

Acid phosphatase. Free acid phosphatase was measured as described by Gianetto & de Duve (1955). Total activities were determined by the addition of 0·1% of Triton X-100

* Part 13: Beaufay, Bendall, Baudhuin, Wattiaux & de Duve, 1959.

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to the reaction medium as described by Wattiaux & de Duve (1956).

Glutamic dehydrogenase. In order to minimize the errors caused by changes in turbidity, free glutamic dehydrogenase was measured by following spectrophotometrically the reduction of cytochrome *c* at 550 mμ in the presence of purified reduced diphosphopyridine nucleotide (DPNH) cytochrome *c* reductase. This has two advantages over following the reduction of diphosphopyridine nucleotide (DPN); in the first place it is more sensitive by a factor of about 6, and secondly the turbidity correction is smaller at 550 mμ than at 340 mμ. For the measurement of free activity the final reaction mixture contained 0·02 M-glycylglycine–NaOH buffer, pH 7·7, 0·03 M-nicotinamide, 0·4 mM-sodium cyanide, mM-EDTA, 0·25 M-sucrose, 0·7 mM-DPN, 0·013 M-potassium L-glutamate, 80 μM-cytochrome *c*, 6 units of reductase and 0·05 ml. of enzyme in a total volume of 1·8 ml. The reaction was started by the addition of enzyme and the increase in extinction at 550 mμ and 25° was followed in a Beckman model D.U. spectrophotometer with a reference cell containing all components of the system except glutamate. For measurement of total activity the reaction mixture was the same but the particle preparation was diluted in a solution containing 0·1% of Triton X-100 and 0·25 M-sucrose. This was preferred to the direct addition of Triton X-100 to the reaction medium since at this concentration the detergent caused some inhibition. Fig. 1 shows that the maximum activity obtained by this method is the same as that obtained by prolonged blender treatment, and it is therefore probable that it gives a true measure of the total activity. In general the sucrose concentration in the reaction medium was always 0·25 M since it caused some inhibition of glutamic dehydrogenase (about 25%). The linear portions of progress curves were used for the calculation of rates. The rate of cytochrome *c* reduction sometimes fell a little at high particle concentrations; to correct for this a calibration curve was always constructed with three different concentrations of Triton-treated particles.

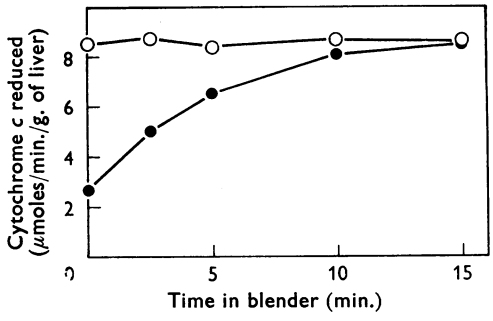

Fig. 1. Comparison of maximum activity of glutamic dehydrogenase obtained by treatment in blender and with Triton X-100. Mitochondria (1 g. of liver/ml.) suspended in 0·25 M-sucrose were diluted 10 times in cold water and treated for various lengths of time in a cooled MSE blender turning at full speed. Sucrose was added to bring the concentration to 0·25 M. Samples were diluted for assay (by the cytochrome *c* method) either in sucrose (●) or in sucrose containing 0·1% of Triton X-100 (○).

The DPNH cytochrome *c* reductase used in these experiments was not very suitable because its relatively high Michaelis constants made it necessary to use high concentrations, and because of the instability of the purified enzyme in solution at 0°. In some later experiments glutamic dehydrogenase was assayed by following DPN reduction at 340 mμ with the same reaction mixture as described above with the omission of cytochrome *c* and reductase. Under these conditions the error caused by turbidity changes was found to be appreciable only when low free activities were being measured. The necessary correction was made by taking readings alternately at 340 and 410 mμ (where DPNH does not absorb). An experimental factor of 1·49 was used to convert E_{410} into E_{340}.

D-*β-Hydroxybutyric dehydrogenase.* This enzyme was assayed by the cytochrome *c* method described above. The reaction medium was the same as for glutamic dehydrogenase except that the substrate was 0·05 M-sodium DL-*β*-hydroxybutyrate. Only the D-isomer will have been oxidized since the L-isomer is oxidized only after conversion into the coenzyme A derivative (Lehninger & Greville, 1953), which could not have been formed under the experimental conditions used. In a few experiments the DPN method was also used. Triton X-100 and a number of other detergents inhibited the enzyme strongly. In order to obtain total activities a suspension in 0·25 M-sucrose was diluted 40 times in water and treated for 10 min. in a cooled blender (Measuring and Scientific Instruments Ltd.) turning at full speed.

L-*Malic dehydrogenase.* The enzyme was assayed by following the reduction of DPN at 340 mμ by the method described by Beaufay, Bendall, Baudhuin & de Duve (1959), except that 0·25 M-sucrose was added to the reaction medium and Triton X-100 was omitted. Total activities were determined by dilution of the particles in a solution containing 0·1% of Triton X-100 and 0·25 M-sucrose.

Materials

Reduced diphosphopyridine nucleotide cytochrome c reductase. The enzyme was purified from pig heart up to the stage R_3 by the method described by Mahler, Sarkar, Vernon & Alberty (1952), with the difference that the first centrifuging was for 5 min. at 1000 *g* only. The final precipitate was dissolved in a 2% (w/v) solution of crystalline bovine serum albumin and stored in small samples in the deep-freeze (−25° approx.). The enzyme was assayed by following spectrophotometrically the reduction of cytochrome *c* at 550 mμ and 25° in a total volume of 1·8 ml. containing 0·03 M-glycylglycine–NaOH buffer, pH 7·7, 80 μM-cytochrome *c*, 0·2 mM-DPNH and enzyme. The reference cell contained all components except enzyme. The unit of activity, defined as change in extinction of 1·00/min. under the above conditions, is slightly different in value from that defined by Mahler *et al.* (1952).

Reagents. Glycylglycine, DPN, cytochrome *c* and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) were obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.), Triton X-100 from Rohm and Haas Co. (Philadelphia, Pa., U.S.A.), L-glutamic acid from the Pfanstiehl Chemical Co. (Waukegan, Ill., U.S.A.), sodium DL-*β*-hydroxybutyrate from British Drug Houses Ltd., L-malic acid from Eastman Organic Chemicals (Rochester, N.Y.) and crystalline bovine plasma albumin from The Armour

Laboratories (Eastbourne). DPNH was prepared by enzymic reduction of DPN as described by de Duve *et al.* (1955).

RESULTS

The activities of the three mitochondrial dehydrogenases were very low in untreated preparations, usually less than 10% of the maximum. They were considerably increased by procedures which activate lysosomal enzymes, namely treatment with detergents, hypotonic conditions, freezing and thawing and mechanical disruption. In general, however, the two types of particle differed in their sensitivity to these treatments.

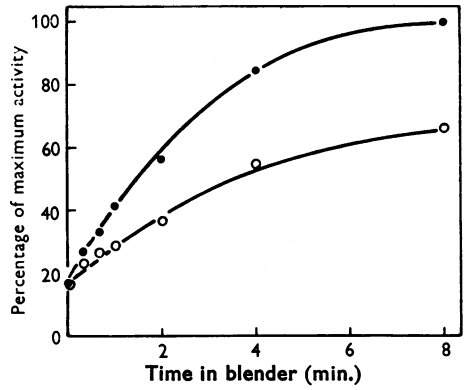

Fig. 2. Activation of glutamic dehydrogenase (●) and acid phosphatase (○) by blender treatment. Samples of mitochondrial fraction (1 g. of liver/5 ml.) suspended in 0·25 M-sucrose were treated for various lengths of time in a cooled MSE blender turning at low speed. Glutamic dehydrogenase was assayed by the cytochrome *c* method.

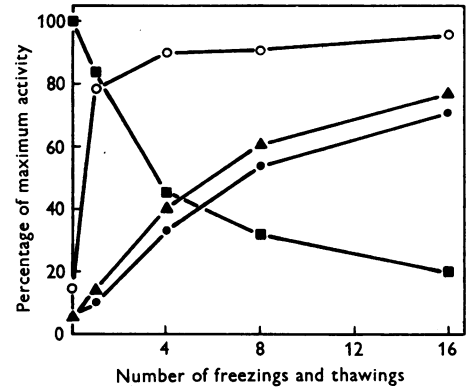

Fig. 3. Activation of glutamic dehydrogenase (●), β-hydroxybutyric dehydrogenase (▲) and acid phosphatase (○) by freezing and thawing. Samples of mitochondrial fraction (1 g. of liver/ml.) suspended in 0·25 M-sucrose in stoppered tubes were frozen in acetone–solid CO_2 and thawed in tap water. Dehydrogenases were assayed by the cytochrome *c* method. ■, Extinction of the suspension at 500 mμ plotted as a percentage of that of the untreated preparation. Extinction of the blender-treated sample used to obtain maximum activity of β-hydroxybutyric dehydrogenase was subtracted from that of the other samples before calculation of the percentage.

Mechanical disruption

Fig. 2 shows the activation of glutamic dehydrogenase and acid phosphatase by treatment of the particles in a blender for various lengths of time. In this experiment the mitochondrial enzyme was more readily activated than the lysosomal enzyme. However, rather variable results have been obtained with blender experiments and the reverse situation has been observed.

Freezing and thawing

Freezing and thawing gave a more clear-cut distinction between the two types of particle (Fig. 3). A few cycles of freezing and thawing gave almost complete release of acid phosphatase. On the other hand, glutamic and β-hydroxybutyric dehydrogenases were considerably more resistant, and even after 16 cycles there was only about 70% activation. In this experiment the decrease in turbidity as a result of freezing and thawing was determined by the change in extinction of the suspension at 500 mμ. The curve obtained showed a reciprocal relation with the activation curve of the mitochondrial enzymes, and clearly was not related to the activation curve of acid phosphatase.

Osmotic activation

Irreversible activation. The irreversible osmotic lysis of mitochondria is shown in Fig. 4. Samples of a particle preparation were diluted in sucrose solutions of decreasing concentration and were allowed to stand in these media for 15 min. at 0°. Sufficient sucrose was then added to return the concentration to 0·25 M and the free activities of the enzymes were determined as soon as possible. Acid phosphatase gave a gradual activation curve which is typical of lysosomes (Appelmans & de Duve, 1955; Gianetto & de Duve, 1955). Glutamic and β-hydroxybutyric dehydrogenases were much more resistant; appreciable activation did not occur until the sucrose concentration was as low as 0·025 M and below that concentration it occurred fairly sharply. The curves for the two dehydrogenases followed each other closely, except at very low sucrose concentrations, where considerably lower activities were obtained for β-hydroxybutyric dehydrogenase. This may be due either to a difference in the intramitochondrial location of the two enzymes, which is suggested by a difference in their solubilities (see below), or to some inactivation of β-hydroxybutyric dehydrogenase at low sucrose

concentrations. In this experiment, the turbidity of the suspension again decreased concurrently with the increase in activity of the dehydrogenases.

The results of a similar experiment are represented in Fig. 5, showing that the activation of malic dehydrogenase also closely follows that of glutamic dehydrogenase. The activities of malic dehydrogenase were actually a little lower but this was probably due to the differences in the conditions for assay of the two enzymes, in particular to the fact that glutamic dehydrogenase was measured by following cytochrome *c* reduction and malic dehydrogenase by following DPN reduction. Greville & Chappell (1959) have shown that the osmotic activation of rhodanese follows a very similar type of curve.

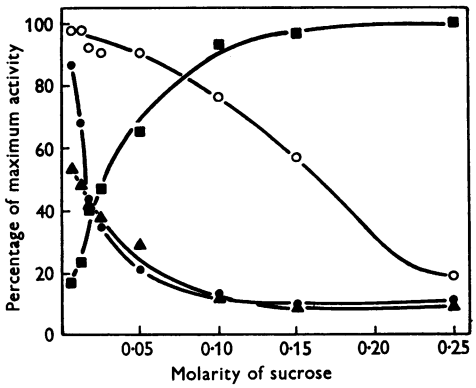

Fig. 4. Osmotic activation of glutamic dehydrogenase (●), β-hydroxybutyric dehydrogenase (▲) and acid phosphatase (○). Preparations were diluted in sucrose solutions of various concentrations. After 15 min. at 0° sucrose was added to bring the concentration to 0·25 M. Dehydrogenases were assayed by the cytochrome *c* method. ■, Extinction of the suspension at 500 mμ measured 9 min. after activation and plotted as a percentage as in Fig. 3.

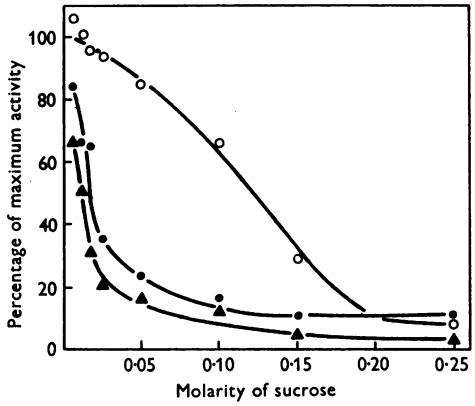

Fig. 5. Osmotic activation of malic dehydrogenase (▲), glutamic dehydrogenase (●) and acid phosphatase (○). Activation was as shown in Fig. 4. Glutamic dehydrogenase was measured by cytochrome *c* reduction and malic dehydrogenase by DPN reduction.

Reversible activation. Irreversible osmotic activation does not occur until the sucrose concentration is as low as 0·025 M. However, reversible osmotic swelling can occur at much higher concentrations (Tedeschi & Harris, 1955), and it was of interest to know whether a reversible osmotic activation of glutamic dehydrogenase occurs at the same time. To test this possibility it was necessary to do the preliminary activation in the reaction medium, except that DPN was not added until the reaction was started. The calculated osmolar concentration of the activation medium was brought to a minimum of 0·0362 M by reducing as much as possible the concentrations of the components of the reaction system, and it was varied by the addition of sucrose. After activation for 5 min. only at 25° the activity of glutamic dehydrogenase was determined either in this same medium with the sole addition of DPN, or after addition of sucrose to bring the concentration to 0·25 M. These results have been plotted in Fig. 6. There was a considerable degree of irreversible activation, which was greater than usual because of the higher temperature of activation. The small difference between the two curves shows that there was only a small degree of reversible osmotic activation.

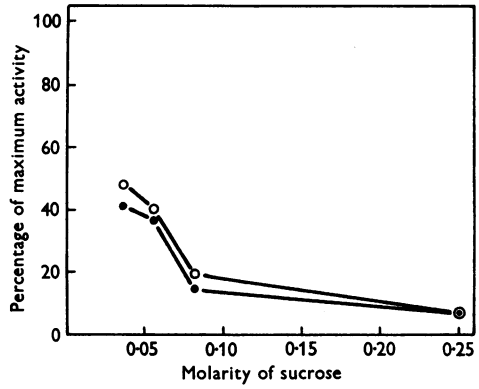

Fig. 6. Reversible osmotic activation of glutamic dehydrogenase. Activation was for 5 min. at 25° in a medium containing 0·01 M-glycylglycine–NaOH buffer, pH 7·7, neutralized 0·4 mM-NaCN, mM-EDTA, 80 μM-cytochrome *c*, pH 7·7, 3 units of DPNH cytochrome *c* reductase/ml., 5 mM-potassium glutamate or 5 mM-KCl (for the blank) and sucrose to bring the osmolar concentration to the value shown on the abscissa. The calculated osmolar concentration of the activation medium without sucrose was 0·0312 M. The reaction was started immediately after activation by the addition of 0·7 mM-DPN with (●) or without (○) the addition of 0·25 M-sucrose. Reduction of cytochrome *c* followed at 550 mμ and 25°.

Table 1. *Solubility of glutamic dehydrogenase after treatment in a blender*

Samples of mitochondria suspended in 0·25 M-sucrose containing mM-EDTA, pH 7·7, (1 g. of liver/2 ml. of suspension), were treated in a MSE blender for the times stated below, diluted twice in sucrose–EDTA and centrifuged for 30 min. at 100 000 *g* in a Spinco model L ultracentrifuge with rotor no. 40. Glutamic dehydrogenase was assayed by the cytochrome *c* method; activities are represented as the percentage of the total activity of the suspension before centrifuging.

Time in blender (min.)	Original suspension. Free	Supernatant. Total	Pellet Total	Pellet Free	Recovery
0	9·1	2·1	99·5	10·0	101·6
15	67·7	73·1	29·3	5·9	102·4
30	75·5	74·9	10·2	4·9	85·1

Table 2. *Solubility of glutamic dehydrogenase after treatment with Triton X-100*

Mitochondria were suspended in 0·25 M-sucrose containing mM-EDTA and various concentrations of Triton X-100 (mitochondrial concn.: 1 g. of liver/2 ml. of suspension). After standing for 15 min. at 0° samples were centrifuged and glutamic dehydrogenase activities were determined as described in Table 1. Figures represent percentages of the total activities of the suspensions before centrifuging (0·1 % of Triton did not cause complete activation because of the high concentration of particles).

Concn. of Triton X-100 (%)	Original suspension. Free	Supernatant. Total	Pellet. Total	Recovery
0	9·3	0·9	101·4	102·3
0·05	4·8	2·3	90·5	92·8
0·1	20·4	5·4	100·0	105·4
1·0	95·8	100	6·0	106·0

Table 3. *Solubility of malic and β-hydroxybutyric dehydrogenase after osmotic activation*

Activation in 0·00625 M-sucrose was carried out as described in Fig. 4. After transfer to 0·25 M-sucrose the free activity was measured on a sample and the remainder was centrifuged for 30 min. at 100 000 *g*. Activities are represented as percentages of the total activities of the original suspension. Assays were by DPN reduction.

	Malic dehydrogenase	β-Hydroxybutyric dehydrogenase
Free activity	73·1	46·5
Soluble activity	32·8	0·3
Particulate activity	81·2	51·0
Recovery	114·0	51·3

Solubility of the enzymes after activation

Activation of glutamic and malic dehydrogenases was found to be associated with a liberation of the enzymes into solution, but β-hydroxybutyric dehydrogenase remained bound to the insoluble residue of the particles. This was investigated by a comparison of the free activity of an activated suspension with the activities in the supernatant and in the pellet after centrifuging the suspension at 100 000 *g* for 30 min. in a Spinco preparative ultracentrifuge. The solubility of glutamic dehydrogenase after blender and Triton X-100 treatment is shown in Tables 1 and 2. In either case, when the free activity was high there was a good correlation between free activity and soluble activity. However, when the free activity was low it was considerably higher than the soluble activity.

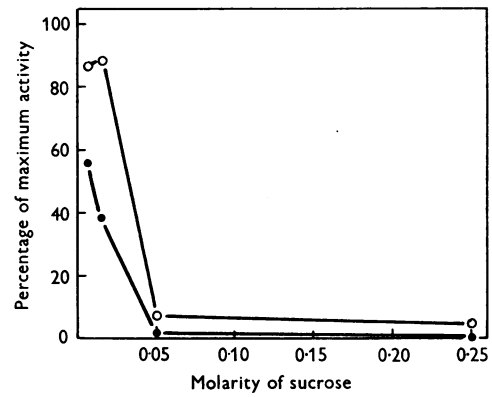

Fig. 7. Correlation of free and soluble activities of glutamic dehydrogenase after osmotic activation. Activation was as in Fig. 4. The activated suspensions (after transferring to 0·25 M-sucrose) were centrifuged for 30 min. at 100 000 *g*. Soluble activity (●) was measured on the supernatant, and free activity (○) on the activated suspension. Assay was by DPN reduction.

The correlation between soluble glutamic dehydrogenase and free activity after hypotonic treatment is shown in Fig. 7. There was an appreciable liberation of enzyme into solution but the soluble activity was distinctly lower than the free activity. The results obtained with malic and β-hydroxybutyric dehydrogenases in the same experiment are shown in Table 3. Malic dehydrogenase behaved like glutamic dehydrogenase; some of the activity was liberated into solution but soluble activity was

lower than free activity. On the other hand, practically all the activity of β-hydroxybutyric dehydrogenase was recovered in the pellet. This result is not conclusive because of the poor recovery, but the insolubility of β-hydroxybutyric dehydrogenase is in agreement with the results of other workers. For example, the phosphorylating sub-mitochondrial particles which have been obtained by digitonin (Cooper & Lehninger, 1956) and sonic (McMurray, Maley & Lardy, 1958; Kielley & Bronk, 1958) treatment of mitochondria retain this enzyme but not glutamic dehydrogenase.

DISCUSSION

Four different treatments have been found to activate the latent glutamic dehydrogenase of mitochondria, in each case up to maximum values of approximately the same magnitude. In several instances, this activation could be correlated with changes in the turbidity of the mitochondrial suspension, with a certain degree of solution of the enzyme and with the simultaneous activation of two other latent dehydrogenases. These facts, together with the varied nature of the activating agents themselves, make it very likely that the latter all act by modifying a structural barrier restricting the accessibility of the enzymes to one or more of their substrates. It has been found by Lehninger (1951) that DPNH is oxidized very slowly by intact mitochondria and the latency of the dehydrogenases under the conditions of our assays is therefore explainable already on the basis of a relative impermeability of the mitochondrial membrane to DPN. However, it is probable that the penetration of the oxidizable substrates is also limited, in view of the low rates of oxidation of these substrates with molecular oxygen as final acceptor (Johnson & Lardy, 1958), as compared with the maximum activity of the dehydrogenases (Beaufay, Bendall, Baudhuin & de Duve, 1959) and of the electron-transport system (de Duve *et al.* 1955).

Several types of activation may be distinguished within the general framework of the above interpretation. The most clear-cut one goes together with a quantitative release of the enzyme molecules in soluble form, as is the case for glutamic dehydrogenase after blender treatment or exposure to high concentrations of Triton X-100. A more complex type is the one affecting glutamic dehydrogenase in the presence of low concentrations of Triton X-100 and both glutamic and malic dehydrogenases after exposure to media of low tonicity. Partial solution of the enzymes occurs under these conditions, but the free activities are higher than the soluble activities. This could be due to the fact that the mitochondrial membrane has been rendered more fragile by the activating procedure and that some of the particles burst when transferred from 0° to the reaction medium at 25°. Alternatively, it is possible that the damage suffered by some of the mitochondria is sufficient to allow the substrates to penetrate into the particles but not to allow the enzymes to leak out of them. That the latter phenomenon may occur, even to some extent in a reversible manner, is suggested by the results of Fig. 6 and by those of Ernster & Navazio (1956), who observed an activation of glutamic dehydrogenase by ageing under aerobic conditions at 30°, which could be reversed by the addition of adenosine triphosphate. It is likely from the work of Greville & Chappell (1959) that the activation of rhodanese is similar to that of the dehydrogenases. These authors have made the interesting observation that agents such as phosphate and thyroxine which induce swelling of mitochondria also cause an activation of rhodanese. The relation between activation and solubility will depend also on the type of link existing between the enzymes and the insoluble framework of the mitochondria. This link appears to be much stronger for β-hydroxybutyric dehydrogenase than for the other two dehydrogenases studied in this work.

Despite these complicating factors, the activation curves of the three mitochondrial enzymes showed a fairly close parallelism, and differed significantly in several cases from the corresponding curves obtained for the lysosomal acid phosphatase. These differences could possibly be due to the fact that acid phosphatase was assayed under more drastic conditions (pH 5·0, 37°) than the dehydrogenases (pH 7·7, 25°). However, this cannot be the only explanation, since the osmotic activation of acid phosphatase has been found to follow the same course, whether the enzyme is assayed at pH 5·0 and 37° or at pH 6·1 and 0° (Appelmans & de Duve, 1955). The results obtained in this investigation may therefore be taken as providing an independent line of evidence for the existence of mitochondria and lysosomes as distinct species of particle. In general, lysosomes appear to be more sensitive than mitochondria to osmotic rupture and to damage by freezing and thawing, but not necessarily to mechanical disruption in a blender.

SUMMARY

1. A comparison has been made between the activation of a latent mitochondrial enzyme glutamic dehydrogenase, and that of a lysosomal enzyme, acid phosphatase, by freezing and thawing, hypotonicity, the neutral detergent Triton X-100 and blender treatment.

2. Malic and D-β-hydroxybutyric dehydrogenases were also found to occur in mitochondria in

a latent form. The activation of these enzymes was compared with that of glutamic dehydrogenase.

3. In graded activation experiments the activation curves for the three dehydrogenases followed each other closely, but they were distinct from the curves for acid phosphatase.

4. A marked irreversible osmotic activation occurred at sucrose concentrations below 0·025 M. At higher sucrose concentrations only a small degree of reversible activation of glutamic dehydrogenase could be observed.

5. Activation led to a liberation of glutamic and malic dehydrogenases into solution, whereas β-hydroxybutyric dehydrogenase remained bound to the particle residue.

6. Activation is interpreted as being due to an increased permeability of the mitochondrial membrane. It is suggested that two types of activation may be distinguished, depending on whether the increase is sufficiently large to allow enzyme molecules to leak out, or only to allow substrate molecules to penetrate. The former kind is irreversible whereas the latter can possibly be reversed.

These investigations have been supported by grants from the Rockefeller Foundation and the Lilly Research Laboratories. One of us (D. S. B.) is indebted to the Belgian Government for a scholarship and to the Administrators of the H. E. Durham Fund, King's College, Cambridge, for a grant.

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Tissue Fractionation Studies

15. INTRACELLULAR DISTRIBUTION AND PROPERTIES OF β-*N*-ACETYLGLUCOSAMINIDASE AND β-GALACTOSIDASE IN RAT LIVER

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(*Received* 5 *August* 1959)

Investigations of the last few years have disclosed that mammalian tissues contain other β-glycosidases besides the well-known and widely studied β-glucuronidase. According to a recent survey by Conchie, Findlay & Levvy (1959), the most active of these is β-*N*-acetylglucosaminidase, followed by β-galactosidase. The intracellular distribution of these enzymes has not yet been studied in detail, but Pugh, Leaback & Walker (1957) have published some preliminary observations indicating that the β-*N*-acetylglucosaminidase of rat kidney is partly associated with subcellular particles and can be released from them by treatment in a Waring Blendor.

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